

# An In Vitro Spinal Cord–Hindlimb Preparation for Studying Behaviorally Relevant Rat Locomotor Function

Heather Brant Hayes,<sup>1</sup> Young-Hui Chang,<sup>1,2</sup> and Shawn Hochman<sup>1,3</sup>

<sup>1</sup>Department of Biomedical Engineering, Georgia Institute of Technology and Emory University; <sup>2</sup>School of Applied Physiology, Georgia Institute of Technology; and <sup>3</sup>Department of Physiology, Emory University School of Medicine, Atlanta, Georgia

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**Hayes HB, Chang Y-H, Hochman S.** An in vitro spinal cord–hindlimb preparation for studying behaviorally relevant rat locomotor function. *J Neurophysiol* 101: 1114–1122, 2009. First published December 10, 2008; doi:10.1152/jn.90523.2008. Although the spinal cord contains the pattern-generating circuitry for producing locomotion, sensory feedback reinforces and refines the spatiotemporal features of motor output to match environmental demands. In vitro preparations, such as the isolated rodent spinal cord, offer many advantages for investigating locomotor circuitry, but they lack the natural afferent feedback provided by ongoing locomotor movements. We developed a novel preparation consisting of an isolated in vitro neonatal rat spinal cord oriented dorsal-up with intact hindlimbs free to step on a custom-built treadmill. This preparation combines the neural accessibility of in vitro preparations with the modulatory influence of sensory feedback from physiological hindlimb movement. Locomotion induced by *N*-methyl *D*-aspartate and serotonin showed kinematics similar to that of normal adult rat locomotion. Changing orientation and ground interaction (dorsal-up locomotion vs ventral-up air-stepping) resulted in significant kinematic and electromyographic changes that were comparable to those reported under similar mechanical conditions in vivo. We then used two mechanosensory perturbations to demonstrate the influence of sensory feedback on in vitro motor output patterns. First, swing assistive forces induced more regular, robust muscle activation patterns. Second, altering treadmill speed induced corresponding changes in stride frequency, confirming that changes in sensory feedback can alter stride timing in vitro. In summary, intact hindlimbs in vitro can generate behaviorally appropriate locomotor kinematics and responses to sensory perturbations. Future studies combining the neural and chemical accessibility of the in vitro spinal cord with the influence of behaviorally appropriate hindlimb movements will provide further insight into the operation of spinal motor pattern-generating circuits.

## INTRODUCTION

The spinal cord contains all the circuitry required for producing the basic rhythmic motor patterns that underlie locomotion. Although this spinal circuitry, often termed the central pattern generator (CPG), can produce locomotor behavior in the absence of phasic sensory input, sensory feedback is known to play an important role in refining the spatiotemporal features of these motor patterns to match environmental demands and correct for unexpected errors. Sensory signals are a major determining factor in both the timing of phase transitions (Duysens and Pearson 1980; Grillner and Rossignol 1978; Hiebert et al. 1996; Pearson et al. 1998; Whelan et al. 1995)

and the magnitude and duration of extensor activity during stance (Hiebert and Pearson 1999; Juvin et al. 2007; Pearson and Collins 1993; Pearson et al. 1998; Rossignol et al. 2006). They are also capable of resetting and entraining centrally generated rhythms in a phase-dependent manner (Conway et al. 1987; Iizuka et al. 1997; Kriellaars et al. 1994; Pearson et al. 1998).

Many of the known properties of the locomotor CPG have been elucidated using the isolated rodent spinal cord, in which the CPG can be robustly recruited through neurochemical application or electrical stimulation. The circuitry can then be dissected anatomically, physiologically, pharmacologically, and, more recently, molecularly with the advent of transgenic approaches. The ability to apply drugs in known concentrations without interference from the blood–brain barrier and to control the ionic composition of the extracellular environment imparts a great advantage over in vivo approaches (Bagust and Kerkut 1981; Smith and Feldman 1987; Smith et al. 1988). Moreover, the mechanical stability of the isolated cord allows for stable intracellular recordings from small neurons (Cheng et al. 2002; Smith and Feldman 1987; Smith et al. 1988; Wheatley et al. 1994), a challenging technical feat in vivo (Eccles et al. 1961) and unprecedented during nonfictive hindlimb locomotion. Although these advantages make the in vitro preparation a powerful model system for investigating neuronal mechanisms of locomotion, the isolated cord fails to incorporate sensory feedback from ongoing limb movements, known to be vital in the patterning of locomotion (Bagust and Kerkut 1981; Pearson 1995; Pearson et al. 1998; Wheatley and Stein 1992). Many of the spatiotemporal features of motor output, such as phase-transition timing and extensor amplitude modulation, are lost in the absence of sensory influences.

The goal of this work was to develop a novel mammalian preparation that combines the neural accessibility and manipulability of in vitro preparations with the modulatory influence of sensory feedback from ongoing, physiologically relevant movement. This requires the retention of dorsal roots and intact hindlimbs as well as an appropriate environment for natural limb stepping, including appropriate orientation relative to gravity and mechanical interactions. Previous work in the amphibian (mudpuppy) forelimb has progressed toward this goal (Wheatley and Stein 1992), but no such mammalian nor hindlimb locomotor preparation exists. Our new preparation is composed of a fully exposed neonatal rat spinal cord with hindlimbs attached. The isolated cord and hindlimbs are ori-

Address for reprint requests and other correspondence: S. Hochman, Department of Physiology, Emory University School of Medicine, Atlanta, GA 30322 (E-mail: shochm2@emory.edu) or Y. H. Chang, School of Applied Physiology, Georgia Institute of Technology, Atlanta, GA 30332 (E-mail: yh.chang@ap.gatech.edu).

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ented dorsal-up in a physiologically appropriate locomotor posture with the limbs allowed to step on a treadmill.

Here we present the first biomechanical characterization of *in vitro* rat hindlimb locomotion, including kinematics from the dorsal-up preparation. We compare these kinematics to those from mechanically similar *in vivo* conditions to provide a framework for understanding their behavioral relevance. We also show electromyography (EMG) from hindlimbs during both dorsal-up and ventral-up *in vitro* locomotion. Although EMGs of the restrained or air-stepping limb have been reported (Atsuta et al. 1990; Kiehn and Kjaerulf 1996), EMGs from appropriately oriented (dorsal-up) and unrestrained hindlimbs during *in vitro* locomotion have not been shown. Finally, to demonstrate the importance of sensory feedback in motor patterning, we compare muscle-activation patterns between dorsal- and ventral-up locomotion and demonstrate the effect of sensory perturbations on muscle-activation patterns and stride timing.

A portion of these results was previously presented in abstract form (Brant and Chang 2006; Brant et al. 2006).

## METHODS

All procedures described here comply with the principles of The Care and Use of Animals outlined by the American Physiological Society and were approved by the Emory University Institutional Animal Care and Use Committee.

## Dissection

Neonatal rats (Sprague–Dawley) postnatal days 1–4 were first decapitated and eviscerated, leaving only the vertebral column, pelvis, and hindlimbs attached to the cord. All skin was removed except that covering the feet. The preparation was then secured in a dissection chamber filled with continuously oxygenated low-calcium, high-magnesium artificial cerebrospinal fluid (aCSF; see *Bathing solutions*). Ventral vertebratomy and dorsal laminectomy exposed the ventral and dorsal sides from the upper cervical transection to the midsacral level. Care was taken to preserve all ventral and dorsal roots to maintain complete hindlimb innervation.

The isolated cord–hindlimb preparations were then transferred to a perfusion chamber designed to allow dorsal-up, unrestrained hindlimb locomotion (Fig. 1, described in the following text). The cords were secured to the Sylgard with insect pins through the remaining ribs in a dorsal-up posture, with the hindlimbs and sacral cord hanging pendant in the caudal portion of the chamber. Cord position was adjusted to approximate physiological locomotor posture.

## Perfusion chamber and treadmill

Rectangular perfusion chambers were constructed from 3-mm-thick transparent Lucite acrylic sheets (GE Polymer Shapes) and filled with Sylgard. A small Sylgard block was then cut from the caudal portion of each chamber to create space for a custom treadmill and unrestrained hindlimb locomotion.

The treadmill belt, composed of a 30-mm-wide  $\times$  130-mm-long polyurethane belt (McMaster-Carr), was mounted around plastic rollers (Tamiya) and two metal shafts. The shafts were drilled perpen-

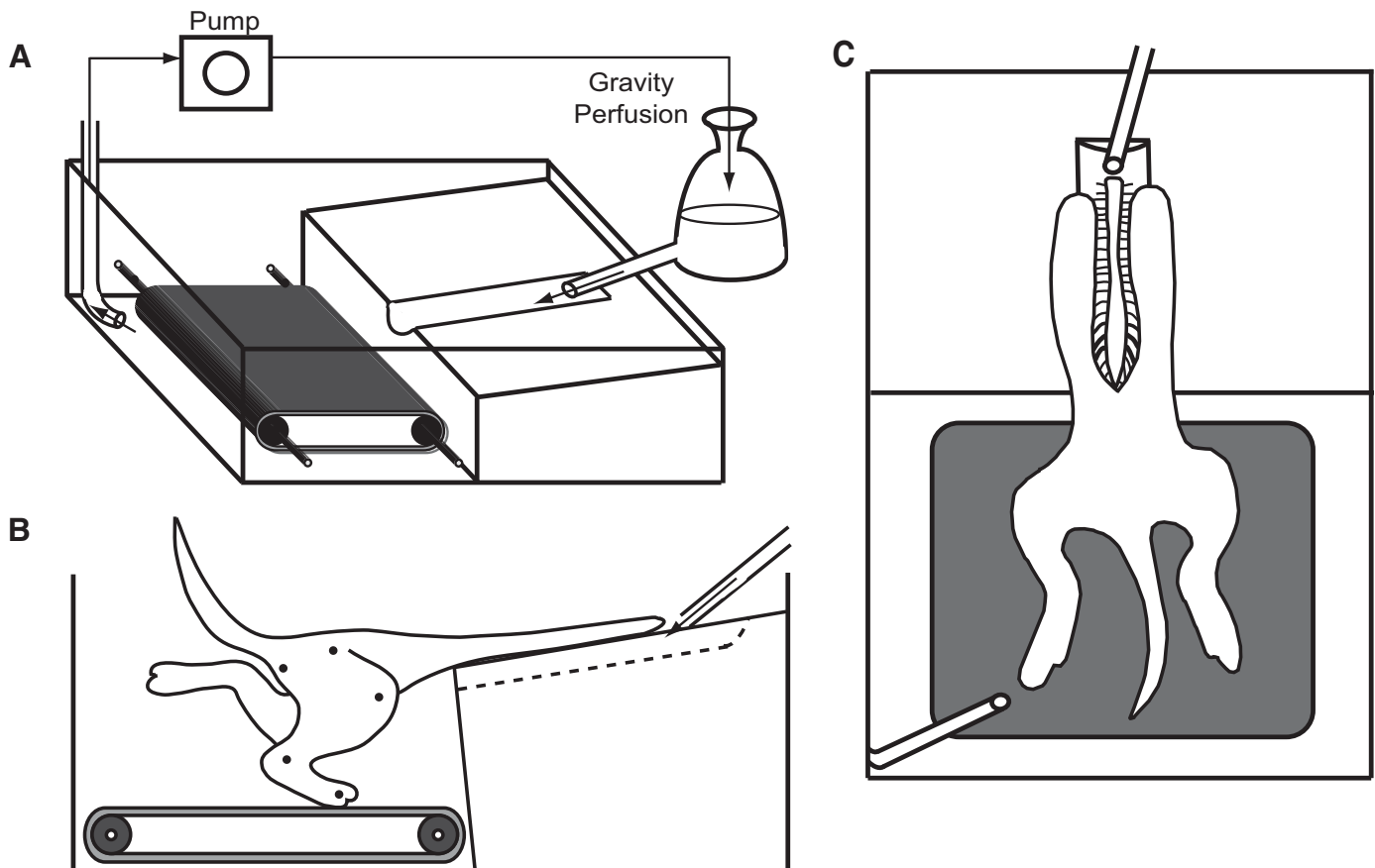


FIG. 1. Experimental setup. *A*: recording chamber and perfusion system. The continuously oxygenated artificial cerebrospinal fluid (aCSF) flows from the gravity perfusion system, through the duct under the ventral surface of the cord, and then is pumped back to the gravity perfusion system via the peristaltic pump. *B*: sagittal view of the isolated cord–hindlimb preparation and treadmill. The exposed cord is secured to the Sylgard by insect pins and the hindlimbs are allowed to locomote freely on a treadmill in the caudal chamber. *C*: overhead view of the isolated cord–hindlimb preparation and treadmill.

dicularly through the Lucite walls and the holes sealed using either epoxy or petroleum jelly to prevent aCSF from leaking from the chamber. The front shaft and roller were driven by a small, brushed DC motor (GM7, Tamiya), whose speed was adjustable between 2 and 12 mm/s by incrementally increasing the voltage across the motor. The back rollers were passively turned by the belt. In later experiments, a brushless DC motor and electronic speed controller (Novak Goat Brushless Crawler System) was used to reduce motor-related noise in the EMG recordings.

Since much of the motor circuitry is known to lie in the ventral portion of the cord, which is partially encased by the remaining vertebral column, a cord perfusion system was added to direct flow along the ventral surface of the cord. A small duct was channeled into the top surface of the Sylgard under the cord. A gravity-fed perfusion system, with the tip of the output at the rostral end of the duct, was then used to supply continuously oxygenated aCSF at 20–30 mL/min, with or without drugs, through the duct beneath the ventral surface. Tubing attached to a peristaltic pump (Cole Palmer Masterflex) was placed in the caudal compartment to recirculate the solution to the gravity system reservoir (Fig. 1A). During initial setup, a biologically inert dye was used to visualize flow and input–output locations were adjusted until diffusion appeared uniform across the ventral surface of the cord. Some experiments were carried out in the absence of the perfusion system, but success of pharmacological activation was greater with continuous perfusion.

### Bathing solutions

All bathing solutions were continuously oxygenated with 95% O<sub>2</sub>-5% CO<sub>2</sub>. The standard bathing solution was aCSF containing (in mM): 128 NaCl, 1.9 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 2.4 CaCl<sub>2</sub>, 1.3 MgSO<sub>4</sub>, and 10 glucose (pH of 7.4). For dissection and EMG electrode insertion, low-calcium, high-magnesium aCSF (same as normal aCSF except for 0.85 mM CaCl<sub>2</sub> and 6.5 mM MgSO<sub>4</sub>) was used to minimize movement. Finally, for pharmacological induction of locomotion, 2–4 μM *N*-methyl *D*-aspartate (NMDA) and 40–60 μM serotonin (5-HT) were added to the aCSF.

### Muscle recordings and analysis

To record EMG activity, monopolar Teflon-coated platinum–iridium electrodes (0.05-mm bare diameter; AM Systems) with bared tips were implanted in up to six hindlimb muscles, including right and left tibialis anterior (TA; ankle flexors), right and left lateral gastrocnemius (LG; ankle extensors), right vastus lateralis (VL; knee extensor), and right rectus femoris (RF; knee extensor/hip flexor). Once implanted, a small drop of formulated cyanoacrylate (Nexaband Liquid Tissue Adhesive; Abbott Laboratories) was placed at the point of electrode insertion to secure the wires during movement. The fine wires were flexible enough to follow the moving limbs with negligible mechanical impedance (Kiehn and Kjaerulf 1996).

The EMG activity was collected through a differential amplifier, band-pass filtered (100–3,000 Hz), notch filtered (60 Hz), digitized at 5 kHz (Digidata 1322A, 16-bit DAQ; Axon Instruments), and recorded (Clampex; Axon Instruments) for off-line analysis. EMG activity was analyzed using a custom program in Matlab (The MathWorks). Analysis relevant to the locomotor rhythm included low-pass Chebyshev filtering to create burst envelopes, burst detection, burst duty cycle, and intermuscular phasing. The phase between two muscles was defined as the time from midburst of the first muscle to midburst of the second divided by the cycle period (Grillner and Matsushima 1992), with 0 being exactly in-phase and 0.5 being 180° out-of-phase. Muscle duty cycles and intermuscular phasing were compared between dorsal-up and ventral-up in vitro preparations and across muscles within a preparation using two-sample and paired Student's *t*-test ( $\alpha = 0.05$ , unless otherwise stated) using the statistics toolbox in Matlab.

### Video recording and kinematic analysis

For sagittal plane kinematic analysis, joint centers were palpated and marked at the hip (greater trochanter), knee (lateral epicondyle), ankle (lateral malleolus), and fifth metatarsophalangeal joints using waterproof black ink. Video recordings of hindlimb locomotion, collected in the sagittal plane using a digital video camera at a rate of 60 Hz, were synchronized with EMG recordings using a trigger light in the camera field of view and a simultaneous voltage pulse sent to an EMG trigger channel. Following collection, joint positions were digitized using semiautomatic tracking (Dartfish Software). Joint angle trajectories for the right hindlimb were then calculated from joint positions. The ankle and knee angles were defined as included angles between the foot and shank segments and shank and thigh segments, respectively. The hip angle was defined as the angle between the thigh and the horizontal. In all cases, increasing angle values indicate extension.

To account for slight variations in cycle time, each step cycle was time normalized. Zero percent gait cycle was defined as the onset of retraction/stance phase, which was determined from video recordings and defined as the time when the toe was in its anterior extreme position (AEP). Similarly, protraction/swing phase onset was defined as the time when the toe was in its posterior extreme position (PEP). Once normalized, joint angular trajectories were averaged across cycles ( $n = 10$ –23 cycles) to compute average trajectories for each animal. Average trajectories  $\pm$  SDs are presented. Stride period was defined as the time from one ipsilateral AEP event to the next, with stride frequency being the inverse of stride period. These and all subsequent kinematic analyses were performed using custom programs in Matlab.

All statistical analyses were performed using JMP statistical software (SAS) or the statistics toolbox in Matlab. Mean stride frequencies at each speed were compared using a one-way ANOVA ( $\alpha = 0.05$ ). The effects of both time and treadmill speed on stride frequency were examined using a multiple linear regression. Coefficients were tested for significant difference compared with zero ( $\alpha = 0.05$ ).

### Ventral-up setup

For comparison, some preparations were oriented ventral-up with their hindlimbs allowed to air-step above the cord. Procedures for ventral-up air-stepping were similar, but were undertaken in a simple flat-bottomed Sylgard chamber. Under these conditions, a static oxygenated aCSF bath was used.

### In vivo kinematics

In vivo kinematics from adult rat treadmill locomotion were also collected. The posterior ischium and hip, knee, ankle, and fifth metatarsophalangeal joints were marked, and video of treadmill locomotion (33.2 cm/s) was recorded at 60 Hz. Sagittal plane kinematics were then analyzed with the same techniques described earlier.

### Success rate

The success rate for dorsal-up in vitro locomotion with the perfusion system was high, with 94% (15/16) locomoting in response to NMDA and 5-HT. However, without the perfusion system, the success rate was much lower at only 39%. For kinematic analysis, some locomoting preparations were excluded because of significant out-of-plane motion or limited paw–treadmill interaction. Also, only kinematics or only EMG activity was collected in some preparations. For ventral-up in vitro locomotion (static bath), the success rate was similarly high, with 84% (13/15) locomoting. Again, some were excluded for out-of-plane motion and some were used only for either kinematics or EMG collection.

RESULTS

*Hindlimb two-dimensional kinematics from the in vitro preparations*

Figure 2 shows average kinematic trajectories for the hip, knee, and ankle joints during locomotion from representative animals in the dorsal-up (Fig. 2A;  $n = 5$ ) and ventral-up (Fig. 2B;  $n = 3$ ) orientations. In the dorsal-up orientation, the hip and ankle extended during retraction/stance and flexed during protraction/swing, as would be expected for stepping in the behaving rat (Gillis and Biewener 2001; Thota et al. 2005). The knee joint exhibited mostly flexion during retraction/stance, presumably supporting the weight of the hindquarters on ground contact as well as actively retracting to accelerate the hindquarters relative to the treadmill. During protraction/swing, the knee exhibited a large extension to return to its initial position.

Overall, the joint trajectories from ventral-up air-stepping differed considerably from those seen in dorsal-up treadmill locomotion. First, the hip and ankle exhibited large plateau phases at the end of retraction/stance phase extension, which

were absent in dorsal-up locomotion. Most interestingly, the knee exhibited a completely different trajectory. Rather than flexing, the knee extended during the entire retraction/stance phase, followed by rapid flexion and slight extension during the protraction/swing phase (Fig. 2B). This difference distinguishes the two movements and results in a very different coordination and phasing between the three joints. Finally, all three joints exhibited a much larger range of motion during ventral-up air-stepping compared with dorsal-up.

*Comparison of kinematics from in vitro and in vivo conditions*

Kinematics from both in vitro conditions (dorsal-up treadmill locomotion and ventral-up air-stepping) compare well with their in vivo counterparts. Figure 2C shows kinematic trajectories obtained during adult in vivo treadmill locomotion. Despite the large age and size differences, the neonatal in vitro dorsal-up treadmill locomotion (Fig. 2A) and adult in vivo treadmill locomotion (Fig. 2C) conditions produced similar kinematics. Neither exhibited the extended plateau phases

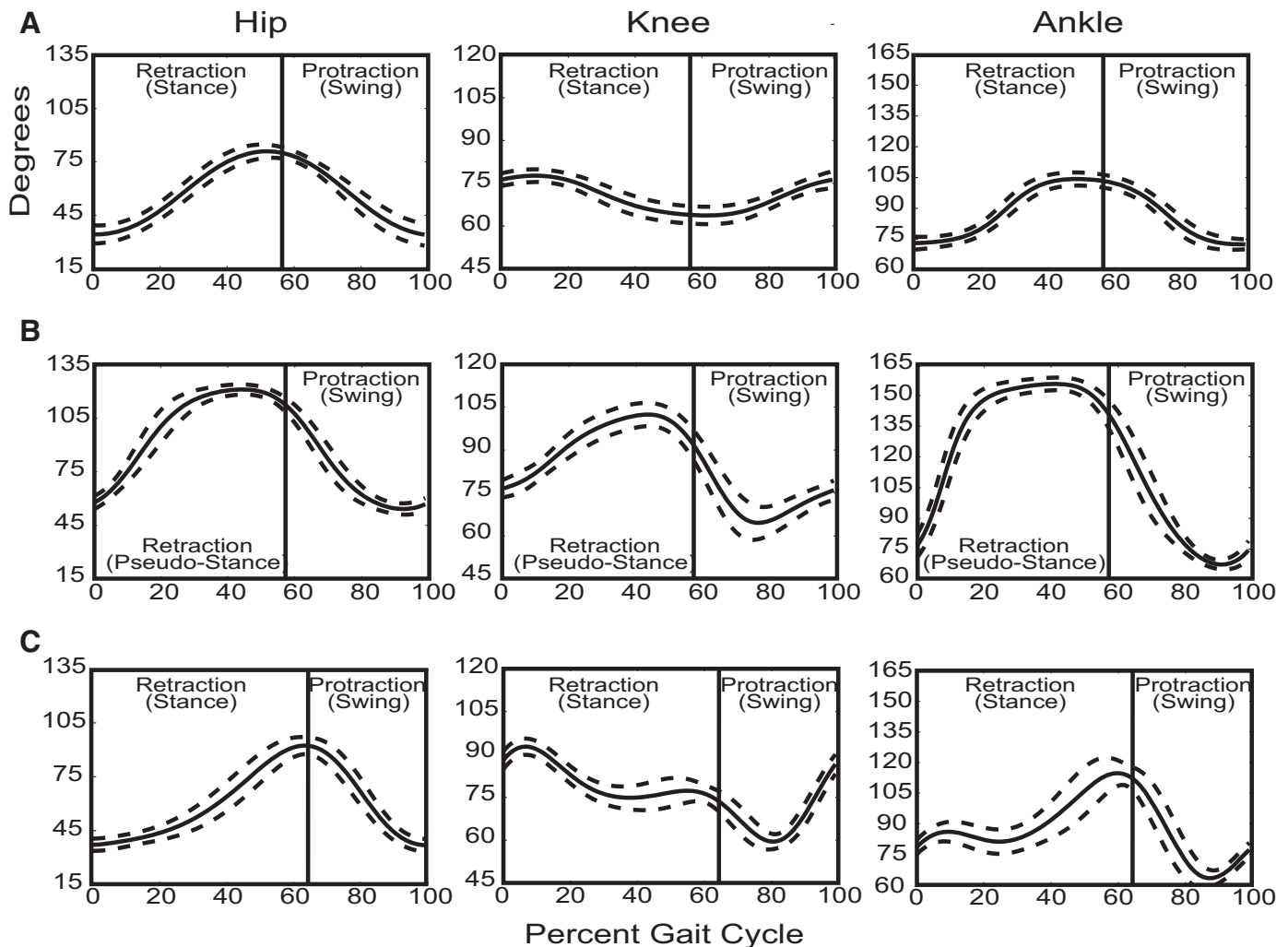


FIG. 2. In vitro and in vivo kinematics. A: dorsal-up in vitro kinematics from locomotion induced by 2–4  $\mu$ M *N*-methyl-D-aspartate (NMDA), 40–60  $\mu$ M serotonin (5-HT). Solid line: average ( $n = 17$  cycles) angular trajectories at the hip (left), knee (center), and ankle (right) plotted over the gait cycle. Dashed lines: average trajectory  $\pm$ 1SD plotted over the gait cycle. 0% gait cycle represents the onset of retraction/stance phase. The solid vertical line represents the average percentage gait cycle at which the onset of protraction/swing phase occurred. B: ventral-up in vitro kinematics from locomotion induced by 2–4  $\mu$ M NMDA, 40–60  $\mu$ M 5-HT (average of  $n = 20$  cycles). C: in vivo kinematics from adult rat during treadmill locomotion (average of  $n = 17$  cycles).

observed during air-stepping and all three joint trajectories followed similar patterns. In both cases, the knee underwent a large flexion phase during retraction/stance that was absent in air-stepping trajectories. It should be noted that there is a difference between the ankle trajectories, with the *in vivo* exhibiting an early flexion phase (sometimes termed E2 phase) during retraction/stance that is absent *in vitro*. The *in vivo* knee trajectory also has an additional flexion phase near the transition to swing that is not present *in vitro*. These absences *in vitro* likely result from the smaller size and added weight support of the Sylgard step. Nonetheless, the differences are small compared with the near reversal in knee joint trajectory between ventral- and dorsal-up *in vitro* conditions.

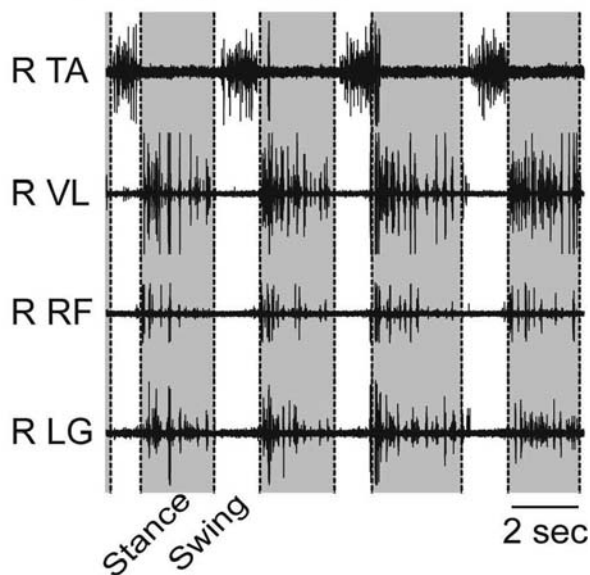
Similarly, the *in vitro* air-stepping trajectories (Fig. 2B) were similar to those previously reported from *in vivo* neonatal air-stepping (Stehouwer et al. 1994). The most obvious similarities include the presence of extension plateau phases at the ankle and hip joints as well as the absence of knee flexion during retraction/stance phases, which reflects the absence of ground interaction and need for weight support.

*Comparison of EMG patterns from the dorsal-up and ventral-up in vitro preparations*

To gain more direct insight into the role of sensory feedback during locomotion, we also looked at EMG activity during dorsal-up and ventral-up *in vitro* locomotion. Figure 3 shows representative EMG activity during locomotion from a dorsal-up and ventral-up *in vitro* preparation. Gray shading indicates retraction/stance phases. In both conditions, the EMG activity showed the expected alternation between ipsilateral ankle flexors and extensors (i.e., right LG and right TA) or between contralateral ankle flexors (i.e., right TA and left TA). However, the intermuscular phasing and relative duty cycles differed significantly between the dorsal-up and ventral-up conditions. First, the phasing between TA, an ankle flexor, and VL, a knee extensor, was significantly different ( $P = 0.002$ ) between dorsal-up ( $0.375 \pm 0.057$ ,  $n = 4$ ) and ventral-up ( $0.0961 \pm 0.101$ ,  $n = 5$ ). The phasing between TA and RF, a knee extensor/hip flexor, showed a similar trend, with the dorsal-up phase ( $0.207 \pm 0.094$ ,  $n = 4$ ) being greater than ventral-up ( $0.164 \pm 0.119$ ,  $n = 4$ ), although this difference was not statistically significant.

Muscle duty cycles also showed significant differences between the *in vitro* conditions. During dorsal-up locomotion, the duty cycles of the extensors VL and LG were  $0.52 \pm 0.064$  and  $0.56 \pm 0.073$ , respectively, whereas the duty cycle of the flexor TA was  $0.37 \pm 0.098$ . The duty cycles of both extensors were significantly longer than the flexor duty cycle (VL,  $P < 0.01$ ,  $n = 4$ ; LG,  $P < 0.05$ ,  $n = 5$ ). During ventral-up locomotion, the duty cycle of VL was  $0.34 \pm 0.12$  and TA was  $0.47 \pm 0.094$ . The ventral-up duty cycles were thus reversed compared with dorsal-up, with the duty cycle of TA being significantly longer than that of VL ( $P < 0.01$ ,  $n = 5$ ). In addition, the duty cycle of VL was significantly longer during dorsal-up locomotion compared with ventral-up ( $P < 0.05$ ), whereas the duty cycle of TA tended to be shorter during dorsal-up locomotion ( $P = 0.06$ ).

**A Dorsal-up *in vitro***



**B Ventral-up *in vitro***

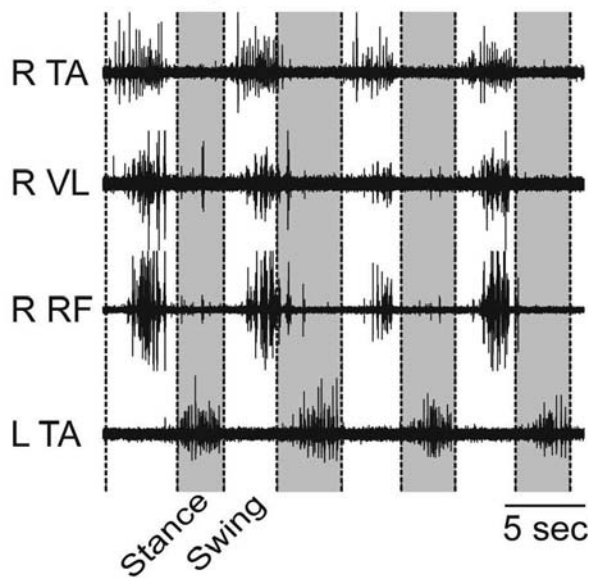


FIG. 3. *In vitro* electromyographic (EMG) activity during (A) dorsal-up and (B) ventral-up locomotion induced by 2–4  $\mu$ M NMDA, 40–60  $\mu$ M 5-HT. Shaded regions represent stance or pseudostance phases and white regions represent swing. Muscles from top to bottom: right vastus lateralis (R VL), right rectus femoris (R RF), right lateral gastrocnemius (R LG in dorsal-up), left tibialis anterior (L TA in ventral-up).

*Effect of sensory perturbations on EMG and stride parameters*

To demonstrate the efficacy of this preparation for studying locomotor circuitry under sensory modulation, EMG activity and stride parameters were examined in response to two sensory manipulations. First, in preparations ( $n = 3$ ) with initially weak locomotion, a stepping assistance perturbation was applied by exerting a swing assistive force on the right hindfoot. A glass probe was used to push the bottom of the right hindfoot anteriorly at the onset of each protraction/swing phase. Figure 4 shows an example of the muscular response to this perturbation. Prior to swing assistance, the locomotion was

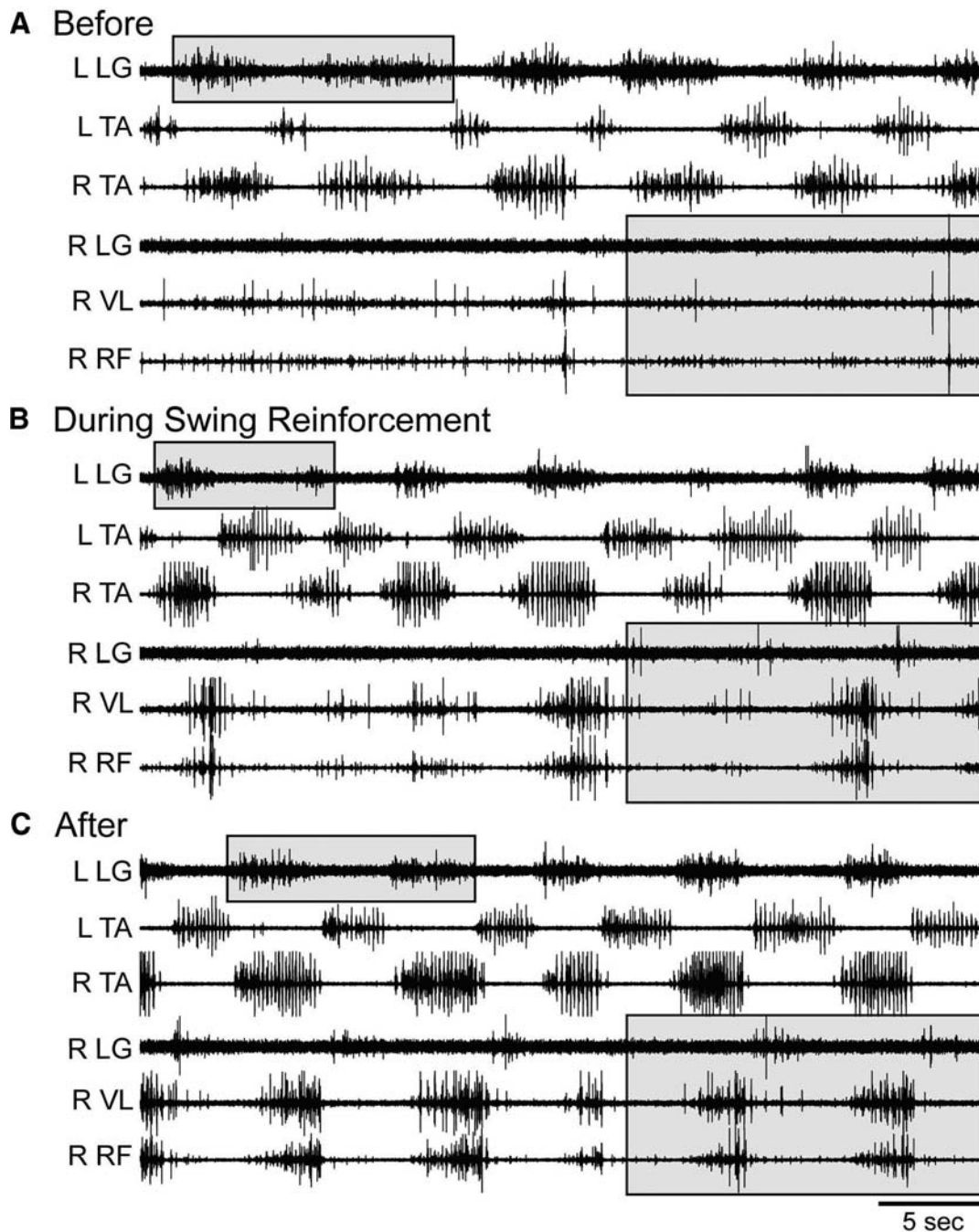


FIG. 4. EMG response to right hindlimb swing assistance. Muscles from *top to bottom* in each panel: left lateral gastrocnemius (L LG), left tibialis anterior (L TA), right lateral gastrocnemius (R LG), right tibialis anterior (R TA), right vastus lateralis (R VL), right rectus femoris (R RF). *A*: 40 s of EMG activity before application of swing assistive forces to the right hindlimb. *B*: 40 s of EMG activity during swing assistance. *C*: 40 s of EMG activity after termination of swing assistance. The *top left* gray boxes in each panel highlight frequency irregularity and/or regularity. The *bottom right* gray boxes in each panel highlight the gradual engagement of the R LG, R VL, and R RF in the rhythm in response to swing assistance.

irregular in frequency and quite weak, with right LG, VL, and RF all failing to burst reliably (Fig. 4A). During swing assistance, the right LG, VL, and RF all began to exhibit regular bursting and engaged in the ongoing rhythmic pattern (Fig. 4B). Finally, after swing assistance was terminated, the EMG pattern was quite robust on all channels and regular in frequency (Fig. 4C). Similar increases in extensor activity strength and regularity were observed in the other two animals. In each case, reinforcing sensory feedback from swing assistance increased the robustness of the centrally generated motor program.

Second, the speed of the treadmill was changed during ongoing locomotion to determine whether this change in sensory feedback could alter hindlimb stride frequency. Such responses have been shown previously in more intact, older animals (Forsberg and Grillner 1973; Grillner and Rossignol 1978; Musienko et al. 2007; Pearson 1995). As treadmill speed increased from about 4 to 11 mm/s, stride frequency increased by 12.1% from a mean of 0.257 to 0.288 Hz (Fig. 5). One-way ANOVA showed that the stride frequencies were significantly different between treadmill speeds ( $P < 0.001$ ). To ensure that

these changes were not simply due to drifts in frequency over time, a multiple linear regression model was used to quantify the effects of both time and treadmill speed on stride frequency. The regression analysis showed that the effect of time was not significant ( $P = 0.5$ ), whereas the effect of treadmill speed was statistically significant ( $P < 0.001$ ). Two other animals also exhibited the trend of increasing stride frequency across a range of treadmill speeds. However, in these two animals, multiple linear regression revealed a stronger influence of time. Due to the compounding factor of temporal drift, the changes in stride frequency could not be attributed to treadmill speed alone. It was noted that the latter two animals appeared to have less forceful interactions with the treadmill belt, whereas the paw of the first animal appeared to cause larger belt deflections during the stance phase. Thus strong paw–belt interaction may be required to achieve a strong speed-entraining effect and alteration of temporal features via belt-related sensory feedback.

## DISCUSSION

The kinematic data presented in this study constitute the first biomechanical characterization of mammalian hindlimb locomotion generated *in vitro*. Importantly, even though locomotion is neurochemically induced and undertaken in neonates, the preparation can produce task-appropriate kinematics and generate locomotor behavior similar to that of the normal adult rat. Although some prior work has described pharmacologically induced “stepping-like movement” in earlier hindlimb-attached *in vitro* preparations (Atsuta et al. 1988; Kudo and Yamada 1987; Smith et al. 1988), no effort has been made to quantify the kinematics of this behavior and thus no data were available for comparison to locomotor behaviors normally exhibited by intact rodents. Additionally, only air-stepping movements have been reported; *in vitro* locomotion has not been studied under behaviorally relevant mechanosensory conditions. The biomechanical characterization presented here also offers a better understanding of the type of behavior being elicited by NMDA and 5-HT acting on the spinal cord and further highlights the role of mechanosensory feedback in altering centrally generated locomotor output. Interestingly, previous EMG activity from restrained hindlimbs suggested that serotonin evokes swimming-like motor output (Kiehn and Kjaerulf 1996). Our results emphasize that mechanosensory input plays a large role in movement patterning and show that 5-HT/NMDA can lead to stepping-like movements when given the appropriate mechanical environment.

### *Kinematics confirm the behavioral relevance of the preparation*

The kinematic dissimilarities between the *in vitro* dorsal- and ventral-up conditions demonstrate the preparation’s ability to produce multiple kinematic behaviors that are largely influenced by the mechanical environment and, likely, sensory feedback. Additionally, they show that the same neurochemicals (i.e., 5-HT and NMDA) can produce different movement patterns depending on the mechanosensory context.

When compared with their *in vivo* counterparts, the kinematics of the *in vitro* preparations strongly resembled the patterns produced in the corresponding mechanical conditions

*in vivo*. The dorsal-up treadmill locomotion joint angle trajectories (Fig. 2A) were comparable to those typically seen in adult treadmill locomotion *in vivo* (Fig. 2C), with the exception of two inflections absent *in vitro*. The slight differences can likely be attributed to discrepancies in age and size and obvious differences between *in vitro* and *in vivo* preparations. Regarding the ventral-up air-stepping (Fig. 2B), the patterns were strikingly similar to the air-stepping patterns previously reported in neonatal *in vivo* air-stepping (Stehouwer et al. 1994). In the *in vivo* study, the intact neonates were harnessed and air-stepping, while *in vitro*, the isolated cord was ventral-up with the hindlimbs air-stepping above the body. Despite this difference in orientation, the absence of ground interaction and correspondingly reduced sensory input appear sufficient to produce comparable kinematic patterns.

Overall, the similarities between *in vitro* and *in vivo* in both the treadmill and air-stepping conditions suggest that spinal motor circuitry, even at an early developmental stage, is capable of producing task-appropriate motor patterns. Previous researchers have questioned the relevance of the neonatal model since neonates at this age do not typically walk (Smith and Feldman 1987). Regardless, when weight supported (i.e., reduced preparation with vertebral support from the Sylgard step), the neonatal spinal cord is capable of producing adultlike movements given sufficient mechanosensory feedback.

### *Sensory input contributes to spatiotemporal features of motor output*

Although the kinematic comparison showed that the mechanical condition can alter kinematic patterns, we cannot distinguish between purely mechanical effects and the effects mediated by sensory feedback pathways using kinematics alone. Thus we compared EMG patterns between the dorsal- and ventral-up *in vitro* conditions to demonstrate the importance of sensory feedback in patterning motor output in the neonate *in vitro*. A limitation of locomotor studies in the neonatal rat spinal cord is the fatigability of afferent synaptic transmission (Lev-Tov and Pinco 1992), which could minimize the contribution of afferent input to ongoing motor output. Afferent activity is capable of resetting locomotion in the neonatal cord (Iizuka et al. 1997; Kiehn et al. 1992), but the contribution of sensory activity to ongoing locomotion remains uncharacterized in the neonate.

We observed significant differences in both muscle phasing and duty cycle between dorsal- and ventral-up patterns, suggesting that sensory feedback from the mechanical condition of the limbs strongly affects the spatiotemporal features of spinal motor output. As described earlier, the VL and TA were more out-of-phase during dorsal-up locomotion compared with ventral-up, with VL being active further into the stance phase during dorsal-up (Fig. 3). During dorsal-up locomotion, the limb extends against a resistance and bears the weight of the hindquarters during stance, leading to greater limb loading and likely excitatory length and force feedback that could increase extensor activity during stance (Pearson et al. 1998; Rossignol et al. 2006). Such feedback would be very small, if present at all, in the ventral-up condition as the limbs are not extending against a surface. Thus this sensory feedback pathway might explain the shift toward increased stance-phase activity of VL during dorsal-up locomotion.

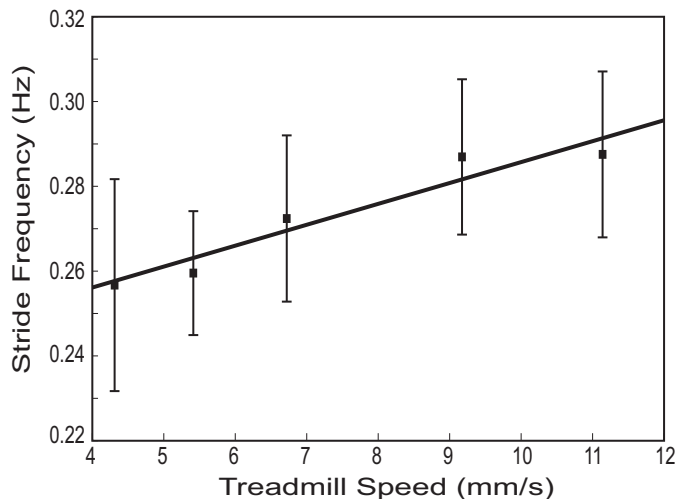


FIG. 5. Stride frequency vs. treadmill speed. Stride frequencies are represented as mean (■)  $\pm$  SD (indicated by error bars) at each treadmill speed. The regression line shows the positive correlation of stride frequency with treadmill speed:  $y = 0.004932x + 0.2364$ .

We further observed a significant difference in relative extensor and flexor duty cycles. During ventral-up in vitro locomotion, the flexor duty cycle exceeded the extensor duty cycle (flexor-dominated; however, see Juvin et al. 2007). During dorsal-up in vitro locomotion, the cycle became extensor-dominated, with the extensor duty cycles exceeding the flexor. Such extensor-dominated muscle activation patterns are typical of intact, behaving mammals, particularly at slower speeds (Engberg and Lundberg 1962; Forssberg and Grillner 1973; Thota et al. 2005). In contrast, the reduction of sensory feedback in the ventral-up in vitro condition (less ground interaction and resistance) likely led to the observed flexor- rather than extensor-dominance in that condition. Similarly, during fictive locomotion where sensory feedback is absent, muscle activation patterns tend to be flexor-dominated as well (71% experiments) (Yakovenko et al. 2005). In sum, these results imply that sufficient and appropriate sensory feedback may be necessary for establishing the extensor-dominance observed during in vivo and dorsal-up in vitro locomotion. The results also emphasize the importance of appropriate sensory feedback for achieving behaviorally relevant locomotor function.

Finally, we considered changes in EMG activity and stride parameters in response to mechanosensory perturbations. First, in response to stepping assistance on the right hindlimb (i.e., applying a swing-assistive force to the limb), the weak and disorganized motor pattern became highly regular and robust across all recorded muscles, showing that appropriate sensory inputs can reinforce ongoing locomotion (Fig. 4). Second, the observed increases in stride frequency with belt speed resulted from changes in sensory feedback, perhaps from stretching of the hip flexors or unloading of the limb, that can initiate the swing phase (Pearson et al. 1998). Together, these responses confirm that sensory feedback can cause major alterations in both the spatial and temporal features of spinal locomotor output in reduced in vitro models, highlighting the importance of retaining appropriate sensory feedback when studying spinal motor circuits in vitro.

### Conclusions and future directions

In conclusion, this model provides a platform for studying the neurophysiology of spinal locomotor circuitry in a behaviorally relevant context, which includes the vital influences of sensory feedback. By bringing the traditional in vitro isolated spinal cord preparation neuromechanically closer to reproducing in vivo locomotion, the dorsal-up in vitro model has the potential to advance the study of neural control of locomotion and the spinal circuits that govern limb movement. The critical interplay between the CPG and proprioception has been the topic of much research, dating back to early commentary by Sherrington near the turn of the century (Mott and Sherrington 1895), but, as discussed in an editorial focus in this journal in 2004, “such questions are difficult to address because they require the analysis of the functional role of neural components in a behaving animal (Cattaert 2004).” The dorsal-up in vitro preparation helps overcome these challenges by allowing us to readily study neural activity with intact sensory feedback from ongoing limb movement.

One of the greatest advantages of the dorsal-up in vitro preparation is the potential for studying interneuronal activity and CPG function during natural, sensory-influenced behavior. As shown in the mudpuppy, once the moving limb is mechanically isolated from the cord, even intracellular recordings can be achieved in in vitro nonfictive preparations (Cheng et al. 2002; Wheatley and Stein 1992; Wheatley et al. 1994). Additionally, the in vitro spinal cord offers exquisite neurochemical and ionic control of the neuronal environment in the absence of a blood–brain barrier. By combining neural accessibility and manipulability with behavioral observability and sensorimotor integration, this novel methodology greatly expands our ability to investigate spinal mechanisms that control locomotion and to elucidate the task-specific functioning of the CPG.

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